Journal of Applied Microbiology 2003, **94**, 207–213

Effects of the antibiotic ionophores monensin, lasalocid, laidlomycin propionate and bambermycin on *Salmonella* and *E. coli* O157:H7 *in vitro**†

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2002/300: received 29 July 2002, revised 21 October 2002 and accepted 29 October 2002

ABSTRACT

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Aims: To examine the effects of ionophores on *Salmonella* and *Escherichia coli* O157:H7 in pure and mixed ruminal fluid cultures.

Methods and Results: Four *Salmonella* serotypes (Dublin, Derby, Typhimurium, and Enteriditis) and two strains of *E. coli* O157:H7 (ATCC 43895 and FDIU 6058) were cultured in the presence of varying concentrations of ionophores (monensin, lasalocid, laidlomycin propionate, and bambermycin) in pure and mixed ruminal fluid cultures. Bacterial growth rates in pure culture were not affected ($P > 0{\cdot}10$) by ionophores at concentrations up to 10 times the approximate rumen ionophore concentration under normal feeding regimens. Likewise, ionophores had no effect ($P > 0{\cdot}10$) on *Salmonella* or *E. coli* CFU plated from 24-h ruminal fluid incubations. Ionophore treatment decreased ($P < 0{\cdot}01$) the acetate : propionate ratio in ruminal fluid cultures as expected.

Conclusions: Ionophores had no effect on the foodborne pathogens *Salmonella* and *E. coli* O157:H7 *in vitro*.

Significance and Impact of the Study: The results suggest that ionophore feeding would have little or no effect on *Salmonella* or *E. coli* populations in the ruminant.

Keywords: anti-microbial, *E. coli* O157:H7, foodborne pathogens, Ionophores, *Salmonella*.

INTRODUCTION

Two of the most important etiologic agents of foodborne illness in humans are *Salmonella* and *Escherichia coli* O157:H7 (Buzby *et al.* 1996). *Escherichia coli* O157:H7, first recognized in 1982, is considered an important agent of foodborne disease with worldwide distribution. The hallmark of infection is a distinctive syndrome characterized by painful, bloody diarrhea with little or no fever, termed hemorrhagic colitis (Griffin and Tauxe 1991; Besser *et al.* 1999). Although outbreaks of *E. coli* in humans have been linked to water, vegetables, fruit juice, and venison, the majority of the cases of human illness for which a source has been determined have resulted from foods that originated from cattle, usually ground beef (Besser *et al.* 1993; Gansheroff and O'Brien 2000; Gage 2001). *Escherichia coli* O157:H7 has been isolated from beef and dairy cattle at all stages of production, and although shedding is intermittent and can be difficult to detect, it appears to be fairly widespread throughout the cattle population (Hancock *et al.* 1998; Elder *et al.* 2000).

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†The research reported was conducted as part of the authors' official duties of the United States Department of Agriculture.

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In humans, *Salmonella* spp. are the most commonly reported and costly cause of foodborne disease in humans with foodborne transmission accounting for approximately 95% of all salmonellosis cases in the United States (Mead *et al.* 1999). *Salmonella* populate the intestinal tracts of various animal species, including beef and dairy cattle, which represent a major reservoir for human foodborne salmonellosis (Fedorka-Cray *et al.* 1998). Beef and dairy products have been identified as important vectors in outbreaks of *Salmonella* in humans (Bean and Griffin 1990).

Ionophores were approved by the United States Food and Drug Administration in the mid-1970s as feed additives for livestock, and since then their use has become routine in the feeding of growing ruminants. The use of ionophores has attracted interest, given the temporal relationship between initial ionophore use in the United States cattle industry and the increase in *E. coli* O157:H7 cases (Griffin and Tauxe 1991; Rasmussen *et al.* 1999). Researchers have suggested that because *E. coli* is a gram-negative bacterium, ionophores might promote the incidence of *E. coli* in cattle by inhibiting competitive gram-positive species (Dennis *et al.* 1981; Henderson *et al.* 1981; Schelling, 1984). However, survey data and experimentation in cattle has yielded conflicting results (Garber *et al.* 1995; Dargatz *et al.* 1997; Herriott *et al.* 1998). In spite of this, the ability of ionophores to alter the gut microbiota may give *E. coli* and/or *Salmonella* a selective advantage and warrants further research. Therefore, a series of experiments were conducted to evaluate the effects of the ionophores monensin, lasalocid, laidlomycin propionate, and bambermycin on four *Salmonella* serotypes and two strains of *E. coli* O157:H7 in both pure and mixed ruminal fluid culture.

MATERIALS AND METHODS

Ionophores

Lasalocid (Bovatec®) and laidlomycin propionate (Cattlyst®) were generously provided by Alpharma, (Chicago Heights, IL, USA). Bambermycin (Gainpro®) was provided by Hoechst Roussel Vet (Warren, NJ, USA). Monensin (Rumensin®) was from Elanco (Greenfield, IN, USA). Stock ionophore solutions were prepared by adding each ionophore to autoclaved 95% (v/v) ethanol in sealed tubes to achieve concentrations of 20, 36, 15 and 2 mg ml^{-1} of monensin, lasalocid, laidlomycin propionate, and bambermycin, respectively. Dilutions from these stocks were made as above to achieve a dose that would approximate ruminal concentrations under normal feeding regimens (0·04, 0·007, 0·003, and 0·0004 mg ml^{-1} rumen fluid of monensin, lasalocid, laidlomycin propionate, and bambermycin, respectively). Multiples of these expected ruminal concentrations (0·125, 0·25, 0·50, 1, 2, 5, and 10×) were made from the stock solutions.

Bacterial cultures

Escherichia coli O157:H7 strain 933 (ATCC 43895) and *Salmonella* serotypes Derby (ATCC 6960) and Dublin (ATCC 15480) were obtained from the American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* O157:H7 strain 6058 (isolated from ground beef following a fatal outbreak of hemorrhagic colitis) was provided by Dr Dan Rice of the Field Disease Investigation Unit at Washington State University in Pullman. *Salmonella* isolates Typhimurium and Enteriditis were obtained from the National Veterinary Services Laboratory (Ames, IA, USA). Strains not naturally resistant to novobiocin (NO) and nalidixic acid (NA) were made resistant to 25 μg ml^{-1} NO and 20 μg ml^{-1} NA. Bacterial strains were cultured in anoxic tryptic soy broth (TSB) medium at 39°C and bacteria that were stable through three successive overnight transfers were utilized in the following experiments.

Pure culture experiments

Pure cultures of *E. coli* O157:H7 strains 933 and 6058 and *Salmonella* serotypes Derby, Dublin, Enteriditis, and Typhimurium were individually added (0·5 ml) to 9 ml of autoclaved TSB. Ionophores were then added (0·2 ml of above solutions) to achieve the desired final concentration. The tubes were sealed, vortexed, and incubated at 39°C. Growth rates were estimated via measurement of optical density (O.D.) at 600 nm using a Spectronic 20D spectrophotometer (Rochester, NY, USA). Cell density readings were taken every 30 min until the O.D. reached 0·6. Each experimental series was replicated three times on separate days.

Mixed culture ruminal fluid experiments

Ruminal fluid was obtained from fistulated Holstein and Jersey cows maintained on a Bermuda grass hay (70%) and concentrate diet. Care, use, and handling of the cattle was pre-approved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA. Ruminal contents were strained via a fine mesh nylon strainer (Reaves and Co., Durham, NC, USA), pooled and transported to the laboratory. Ruminal fluid was transferred anerobically to a medium containing (l^{-1}): 292 mg of $K_2HPO_4.3H_2O$, 240 mg of KH_2PO_4, 120 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4.7\ H_2O$, 64 mg of $CaCl_2.2H_2O$, 600 mg of cysteine hydrochloride, 4 g of Na_2CO_3 and 1 g each of sigmacell, glucose, xylose, and cellobiose. The final concentration of ruminal fluid was 33% (vol/vol). To this ruminal fluid medium, 2 ml of a 1 : 10 dilution of the bacteria was added (initial bacterial concentrations were approximately 10^5 and 10^4 CFU ml^{-1} of *E. coli* and *Salmonella,* respectively).

One millilitre samples of this inoculated ruminal fluid were serially diluted (in 10-fold increments) in phosphate buffered saline (PBS, pH 7·0), plated, and incubated overnight at 37°C for direct counting to determine intitial bacterial concentrations. *Escherichia coli* O157:H7 strains 6058 and 933 were plated on MacConkey's agar supplemented with 25 μg ml^{-1} NO and 20 μg ml^{-1} NA. *Salmonella* serotypes were plated on brilliant green agar (BGA) supplemented with 20 μg ml^{-1} NO.

Ten millilitre of the above medium was added to anoxic tubes flushed with O_2 free CO_2 and the tubes sealed with butyl rubber stoppers and aluminum crimps. The respective ionophores were added (0·2 ml) and tubes incubated for 24 h at 37°C. Following incubation, 1 ml was removed from each sample for serial dilution and plating as described above. Bacterial colonies were directly counted the following day. After plating, diluted samples (1 : 10) were centrifuged, filtered and stored at −20°C until analyzed for volatile fatty acids (VFA) by gas-liquid chromatography as previously described (Corrier *et al*. 1990). This experiment was replicated twice with each bacteria, for all concentrations of each ionophore.

Reagents and supplies

Unless otherwise noted, all media and agar were obtained from Difco Laboratories (Detroit, MI, USA). Reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Data from multiple repetitions were pooled prior to analysis. Growth rates, CFU, and VFA data were subjected to analysis of variance appropriate for a completely randomized design. Analysis of VFA data showed no differences within individual bacterial strains or serotypes; therefore, the two *E. c*oli strains and the four *Salmonella* serotypes were pooled and analyzed collectively as *E. coli* or *Salmonella*. Differences among mean values were considered significant at a 5% ($P < 0·05$) level of significance.

RESULTS

Pure culture experiments

Growth rates for *E. coli* O157:H7 strains 933 and 6058 and for *Salmonella* serotypes Derby, Dublin, Enteriditis, and Typhimurium are presented in Fig. 1. No differences ($P > 0·10$) were found in growth rates for either strain of *E. coli* O157:H7 when comparing increasing concentrations of the same ionophore or when comparing different ionophores. Similarly, the type of ionophore or ionophore concentration had no effect ($P > 0·10$) on growth rate of any of the *Salmonella* serotypes examined. Laidlomycin propionate appeared to increase the growth rate of *Salmonella* dublin compared with other ionophore treatments; however, this effect was not statistically significant.

Mixed culture experiments

Colony forming units of *E. coli* O157:H7 and *Salmonella* after incubation with ruminal fluid are presented in Fig. 2. Increasing ionophore concentrations had no effect ($P > 0·10$) on *E. coli* O157:H7 strain 933 and 6058. Likewise, no differences ($P > 0·10$) were observed when different ionophores were compared. *Salmonella* serotypes were unaffected ($P > 0·10$) by ionophore concentration or type. The highest lasalocid concentration appeared to decrease CFU of *E. coli* strain 933, however this was not statistically significant. In general, *Salmonella* serotypes did not appear to survive as well in rumen fluid incubations, as suggested by lower CFU when compared with *E.coli,* however initial concentrations of inoculated *Salmonella* were one $\log_{10}$ CFU lower. Laidlomycin propionate appeared to decrease CFU of *Salmonella* Derby but this was not significantly different from other ionophore treatments.

Individual analysis of bacterial strains indicating monensin treatment resulted in a numerical, but not significant ($P > 0·10$), decrease in the acetate : propionate ratio (data not shown). When strains 933 and 6058 were pooled and analyzed collectively as *E. coli,* the acetate : propionate ratio decreased ($P < 0·05$) in monensin treatments (Fig. 3). Similarly, when *Salmonella* serotypes were pooled, acetate:propionate ratio decreased in monensin ($P < 0·01$) and laidolomycin propionate ($P < 0·05$) treatments, and tended to decrease ($P = 0·07$) in lasalocid treatments. Bambermycin had no effect ($P > 0·10$) on acetate : propionate ratios (Fig. 3).

DISCUSSION

Ruminant animals are asymptomatic carriers of *E. coli* O157:H7 and other enterohemorrhagic *E. coli* (Beutin *et al.* 1993; Rasmussen *et al.* 1993; Bielaszewska *et al.* 2000; Cornick *et al.* 2000) with the majority of human outbreaks linked to contact with ruminant animals or to products derived from ruminants (Gage 2001). Ninety-five percent of an estimated 1·4 million non-typhoidal *Salmonella* cases in the United States are estimated to be foodborne (Tauxe 1991; Mead et al. 1999) with beef, lamb, and dairy products listed as major sources of infection (Holmberg *et al.* 1984; Bean and Griffin 1990; Cullor 1995). In the United States, ionophores are widely used in the feeding of growing beef

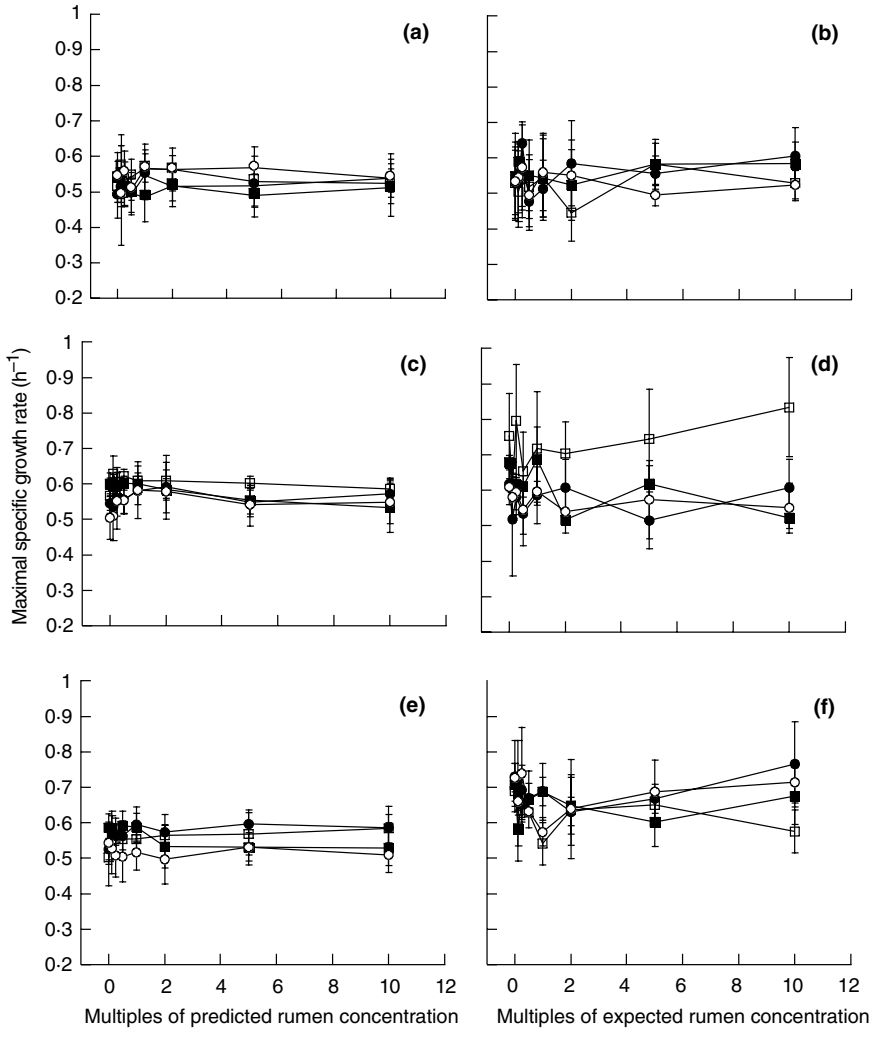

Fig. 1 Specific maximal growth rates (h^{-1}) of *E. coli* O157:H7 strains 933 (a) and 6058 (b) and *Salmonella* serotypes Derby (c), Dublin (d), Enteriditis (e), and Typhimurium (f) after exposure to increasing concentrations (0, 0·125, 0·25, 0·50, 1, 2, 5, and 10×) of lasalocid (■), laidlomycin propionate (□), bambermycin (●) and monensin (○) in pure culture. Base concentration approximates ruminal concentrations under normal feeding regimens (1X = 0·004, 0·007, 0·003, and 0·0004 mg ml^{-1} rumen fluid of monensin, lasalocid, laidlomycin propionate, and bambermycin, respectively)

and dairy cattle, sheep and goats and the benefits to growing ruminants and the subsequent effects of ruminal fermentation are well-documented (Russell and Strobel 1989). The use of ionophores has attracted interest because of the increase in human *E. coli* O157:H7 cases and the corresponding timeframe of widespread ionophore use (Griffin and Tauxe 1991; Rasmussen *et al.* 1999). Furthermore, it is hypothesized that the increasing number of antimicrobial-resistant *Salmonella* strains isolated from human salmonellosis cases are because of widespread use of antimicrobial agents in food animal production and that these resistant strains originate from animals (Cohen and Tauxe 1986). However, to our knowledge, no direct link has been established between ionophore use in ruminants and the development of antimicrobial resistance in foodborne pathogens. In fact, research conducted in our laboratory showed no increase in antimicrobial susceptibility of *E. coli* O157:H7 or *Salmonella* Typhimurium in lambs fed ionophores (T.S. Edrington unpublished data). Bambermycin has been reported to actually decrease antimicrobial resistance of *E. coli* and *Salmonella* in calves and swine (Federic and Sokol 1973; Sokol *et al.* 1973; Dealy and Moeller 1976, 1977).

In our experiments, the ionophores rumensin, lasalocid, and laidlomycin propionate decreased the acetate : propionate ratio in mixed ruminal fluid incubations as expected, a typical response in animals fed ionophores (Russell and Strobel 1989). Overall, we saw no effects of ionophore treatment on *E. coli* O157:H7 or *Salmonella* with respect to growth rates in pure culture or CFU in mixed ruminal fluid incubations. In contrast, the prevalence of *E. coli* O157 was higher in dairy herds that used monensin, lasalocid, and or decoquinate in their heifer rations compared with herds not using these additives (Herriott *et al.* 1998). Supporting our findings, Garber *et al.* (1995) reported no association between fecal shedding of *E. coli* O157:H7 and ionophore use in dairy calves. Dargatz *et al.* (1997) likewise reported no relationship between ionophore use and *E. coli* O157:H7 in

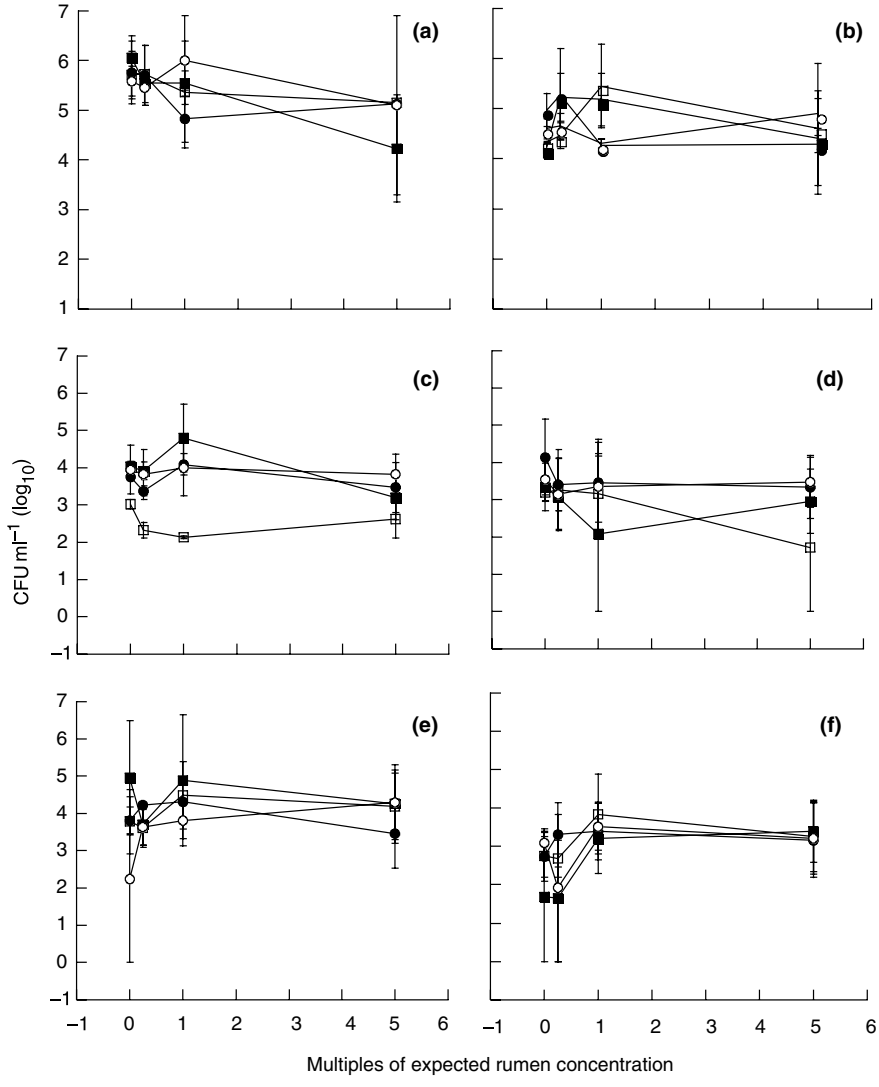

Fig. 2 Colony forming units ($\log_{10}$) of *E. coli* O157:H7 strains 933 (a) and 6058 (b) and *Salmonella* serotypes Derby (c), Dublin (d), Enteritidis (e), and Typhimurium (f) after exposure to increasing concentrations (0, 0·125, 0·25, 0·50, 1, 2, 5, and 10×) of lasalocid (■), laidlomycin propionate (□), bambermycin (●) and monensin (○) in mixed ruminal fluid culture. Base concentration approximates ruminal concentrations under normal feeding regimens (1X = 0·004, 0·007, 0·003, and 0·0004 mg ml^{-1} rumen fluid of monensin, lasalocid, laidlomycin propionate, and bambermycin, respectively)

feedlot cattle. In a survey of 100 United States feedlots, Losinger *et al.* (1997) found no difference in the number of fecal samples positive for *Salmonella* when ionophores were included in the diet. In further support of our results, Dealy and Moeller (1977) reported calves supplemented with bambermycin in their feed had similar intestinal *E. coli* populations compared with control calves. Bambermycin supplemented feed reduced the duration and prevalence of *Salmonella* Typhimurium shedding in experimentally infected calves and swine (Dealy and Moeller 1976, 1977).

Research examining the effects of ionophores on *E. coli* and *Salmonella* are conflicting and highlight the complexity of the ruminant animal. We initiated our research in pure culture to eliminate the confounding effects of the ruminal microflora. The lack of any ionophore effect may be attributed to the double membrane present on gram-negative bacteria that is capable of excluding a variety of compounds (Ahmed and Booth 1981; Brock *et al.* 1994). However, because it is generally accepted that ionophores inhibit gram-positive bacteria favoring gram-negative species (Dennis *et al.* 1981; Henderson *et al.* 1981; Schelling 1984), ionophore feeding could theoretically increase populations of *E. coli* by inhibiting competing gram-positive species. Ionophore exposure may have inhibited gram-positive species in these mixed ruminal culture experiments, however based on our results, it did not benefit *Salmonella* or *E. coli* populations. Disseminating the factors involved in the carriage and shedding of foodborne pathogens in ruminants is a complex, but necessary task, as long as there is opportunity for contamination of our food supply. Incorporating knowledge from this work and others, along with pre- and post-harvest intervention strategies may help to improve the safety of ruminant-derived foods.

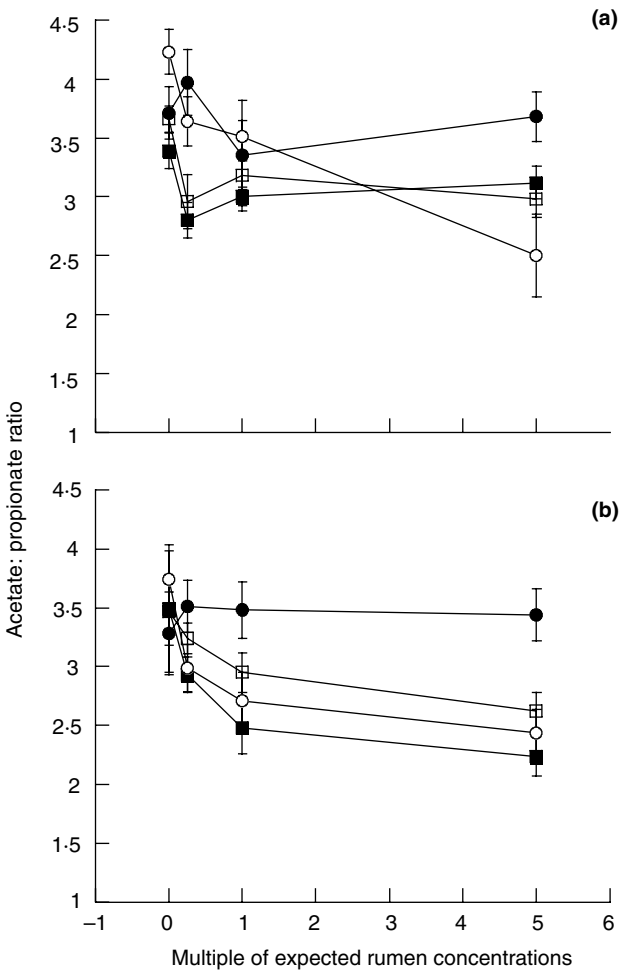

Fig. 3 Pooled acetate : propionate ratios for two strains of *E. coli* O157:H7 (a) and four *Salmonella* serotypes (b) after exposure to increasing concentrations (0, 0·125, 0·25, 0·50, 1, 2, 5, and 10×) of lasalocid (■), laidlomycin propionate (□), bambermycin (●) and monensin (○) in mixed ruminal fluid culture. Base concentration approximates ruminal concentrations under normal feeding regimens (1X = 0·004, 0·007, 0·003, and 0·0004 mg ml^{-1} rumen fluid of monensin, lasalocid, laidlomycin propionate, and bambermycin, respectively)

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Alpharma for generously providing the laidlomycin propionate and lasalocid, and Hoechst Roussel Vet for supplying bambermycin.

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